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Development of a novel diagnostic assay for the diagnosis of canine immune-mediated thrombocytopenia (IMT)

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Clinical abstracts from BSAVA Congress 7–10 April 2016

Clinical Abstracts: PetSavers Grant Awards

Investigating vitamin D metabolism in cats with tuberculosis caused by infection with *Mycobacterium bovis* and *Mycobacterium microti*

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Aims of study: To determine whether infection with *M. bovis* or *M. microti* affects vitamin D receptor levels and if this correlates to more severe clinical signs and disease progression. Understanding the role of the immune response, particularly of macrophages and vitamin D, may help to determine more effective methods for treating cats with mycobacterial infections, or improve diagnosis.

Methods: Twelve feline tissues samples were divided into three groups: *M. bovis*, *M. microti* and 'Healthy'. Four tissue samples in each category were used for this study. Immunohistochemistry was performed to detect the antigens of interest (macrophage specific molecule and vitamin D receptors [VDR]). Antigen retrieval was required to unmask the antigens. Two different methods were used: Heat-induced epitope retrieval (HIER) and Protease-induced epitope retrieval (PIER). Haematoxylin and eosin staining was performed to enable comparison of tissue morphology, including the identification of cellular infiltrates and granulomas. Ziehl–Neelsen (ZN) staining was used to identify acid-fast organisms, primarily mycobacteria.

Results: In *M. bovis*-infected cats granulomas and/or areas of significant inflammatory infiltrates were observed which were associated with the presence of macrophages. These were often clustered with ZN+ organisms. The more macrophages present, the lower the number of bacteria. It was difficult to draw a solid link between expression of VDRs and the severity of disease in the *M. bovis*-infected cats. In *M. microti*-infected cats a link between VDRs and the severity of disease could not be drawn and there was no clear correlation between macrophage and mycobacteria numbers. In the healthy cats there were considerably less VDRs and macrophages than in the cats with mycobacterial infections. Low numbers of macrophages were seen and no ZN+ organisms were identified.

Conclusion: The mycobacteria-infected cats could have up-regulated the number of VDRs present in their tissues in order to increase the responsiveness to vitamin D. It is known that VDRs are up-regulated by 1,25(OH)₂D and since infection increases the conversion of inactive 25(OH)D to active 1,25(OH)₂D this could explain the increased VDR expression seen. Cytokines secreted by T cells during inflammation also affect VDR expression illustrating that regulation of the VDR level is a common mechanism used in the defence against pathogens.

To enhance this study a larger sample size should be used to increase the reliability of the results obtained.

The effects of the different treatment regimes could be accounted for by using a larger sample size and cross referencing the results to the specific treatments. More investigation into vitamin D needs to be carried out in order to gain a better understanding of the effects vitamin D has on the immune response. Researchers have already discovered that higher levels of vitamin D are linked to better survival chances for hospitalised cats. We know that vitamin D plays a major role in the immune response but we need to know how significant that role is in mycobacterial infections; as this could lead to new treatment methods and help fight infections.

Development of a novel diagnostic assay for the diagnosis and monitoring of canine immune-mediated thrombocytopenia (IMT)

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Aim: The aim of this study was to develop a novel diagnostic assay for the diagnosis and monitoring of canine immune-mediated thrombocytopenia (IMT). This is a common condition affecting dogs for which there is no diagnostic test currently available in the UK. Previously described assays used for research purposes require immediate analysis of samples, therefore an additional aim was that the assay must be accurate even when samples are stored for 48 hours at room temperature to mimic postage of samples to a reference laboratory.

Method: A flow cytometry assay was developed utilising 'direct to blood' (DtB) immunofluorescence staining of whole blood at the point of collection. Participating small animal hospitals were provided with pre-prepared sample tubes allowing immunofluorescence staining anti-canine-CD61-Alexa Fluor 647 (to detect platelets), anti-canine IgG-FITC (to detect auto-antibody), thiazole orange (to detect reticulated platelets). After 30 minutes 2% paraformaldehyde was added to fix the sample.

Samples were analysed by two-colour flow cytometry (FACS Calibur) to detect platelets within whole blood and to determine the percentage of platelet surface-associated IgG (PSA-IgG) and the percentage of reticulated platelets (RP). Samples were analysed on the day of collection and after 48 hours to determine whether time had an effect on the results. Platelet counts were measured using a haemocytometer.

Results: Samples were received from 24 dogs (10 healthy, 11 hospitalised and 3 IMT cases).

(i) **Platelet Count:** RP count increased as platelet count decreased.

(ii) **PSA-IgG:** IMT cases had significantly higher percentage of PSA-IgG than healthy or hospitalised dogs ($P = 0.02$).

(iii) **RP:** Both IMT cases and hospitalised dogs had a significantly higher percentage of RP than healthy dogs ($P = 0.0002$).

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(iv) **Comparison of Results at 24 and 48 hours:** The percentage of PSAIgG and RP showed no significant variation over time.

Conclusion: Dogs with IMT have significantly higher percentage of PSAIgG than hospitalised or healthy dogs, and this assay is a potential diagnostic tool for IMT. Furthermore, the technique was robust with analysis >48 hours after sample collection and may therefore be suitable for samples sent by post. The DtB staining kit was simple and easy to use for practitioners and had the added advantage that only a very small volume (<100 µl) of blood is required. Further studies are needed to optimise the assay including analysis of a greater number of samples from varied cases. Additionally, further studies will investigate the effects of existing and novel immunosuppressive agents on PSAIgG and RP.

This project was funded by a BSAVA PetSavers Student Research Grant and a BBSRC Small Project Grant. Heather Birrell was funded by a scholarship from the Carnegie Trust.

Investigation of sensitivity and specificity of DGGR lipase for diagnosis of acute and chronic pancreatitis in dogs

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Pancreatitis is a common, potentially fatal condition of dogs. The DGGR lipase catalytic assay is selective for lipase of pancreatic origin. The test is cheaper than Spec cPL and can be added to in-house biochemistry panels. This study investigated the performance of the DGGR lipase assay for diagnosis of histopathologically confirmed canine acute and chronic pancreatitis.

Agreement between DGGR lipase and Spec cPL assays was also assessed. Dogs with histologically confirmed acute pancreatitis (n = 2), chronic pancreatitis (n = 7) and normal pancreatic tissue (n = 7), that had stored (−80°C) serum samples available, were identified. Sections were reviewed by one author and disease status confirmed. The control population was selected based on clinical signs for which pancreatitis was a differential diagnosis. Azotaemic cases were excluded. DGGR lipase was measured in-house using a previously validated colourimetric DGGR lipase assay. Spec cPL was measured by IDEXX Laboratories.

Sensitivity and specificity of DGGR lipase and Spec cPL were compared using receiver operator characteristic (ROC) curve analysis. Agreements of each assay with histopathology, and with each other, were assessed using Cohen's kappa coefficient (κ). The DGGR lipase assay was 100% sensitive and 100% specific for cases of histologically confirmed acute pancreatitis when a cut point of >190 IU/L was used. Spec cPL was also 100% sensitive and specific for acute cases at a cut point of >200 µg/L. There was a perfect agreement ($\kappa = 1.00$) between histologically confirmed cases of acute pancreatitis with DGGR lipase >190 IU/L and Spec cPL >200 µg/L.

Sensitivity and specificity of the DGGR lipase assay at cut point >190 IU/L was poor (29%) and excellent (100%) respectively for chronic pancreatitis. Sensitivity of Spec cPL was also low for chronic pancreatitis

(29% at cut point >200 µg/L). Agreement between histopathologically confirmed chronic pancreatitis with both lipase assays was fair ($\kappa = 0.286$) at cut points of >190 IU/L for DGGR and >200 µg/L for Spec cPL.

Perfect agreement ($\kappa = 1.00$) was found between DGGR lipase (>190 IU/L) and Spec cPL (>200 µg/L).

The results of this study suggest that the DGGR lipase assay is very sensitive and specific for histopathologically confirmed acute pancreatitis, however it has poor sensitivity for chronic pancreatitis. The DGGR lipase and Spec cPL assays can be used interchangeably allowing a quicker, cheaper and more accessible diagnosis.

A comparison of culture vs 16S ribosomal RNA sequencing of chronic granulation tissue microbiota in cats and dogs

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Background: Chronically healing wounds affect many animals seen in general practice. Routinely, microbiological culturing is used to profile the bacterial species present in these wounds. Studies into the microbiota of these types of wounds in human medicine have shown that culture may be insufficient for detecting the extent of the bacterial species present. Culture-free 16S ribosomal RNA (rRNA) sequencing, being more sensitive than culture, may give a more accurate picture of the bacteria present. This study compares the results of culture and molecular testing in chronic wounds in animals.

Method: Samples from 10 chronic wounds (six dogs and four cats) underwent culture and 16S rRNA sequencing. Genomic DNA was isolated and the 16S rRNA V3-V4 region was amplified by PCR. Subsequently, libraries of the genomic DNA were constructed for sequencing on the Illumina MiSeq platform. The resulting sequence reads were trimmed from primer sequences, paired-end reads were then joined and quality filtered. QIIME was used to cluster reads against the Greengenes database to provide taxonomic assignment, referred to as closed Operational Taxonomic Units (OTU) picking.

Results: Culturing identified six genera across five samples; five samples were culture negative. In contrast, 98 unique genera were identified by 16S rRNA sequencing. *Campylobacter* spp. had the highest overall abundance however it was not detected by culturing in any of the samples, likely due to its growth requirements not being met using standard culturing procedures. Of the six genera identified by culturing, three could be classified to the genus level by 16S rRNA sequencing and two were assigned to family level. One case, which had a history of myiasis, showed a significantly different make-up of the microbiota detected by 16S rRNA sequencing.

Conclusions: The discordance between the culture and 16S rRNA sequencing was dramatic, with culture failing to identify the majority of the microbiota identified by sequencing. This suggests that culturing techniques may be inadvertently selecting for specific bacteria that are easily cultured rather than identifying the most abundant or clinically relevant bacteria. This may, in turn,

be directing veterinarians towards the use of inappropriate antibiotics. It is, however, unclear as to whether some of the detected DNA may be from dead bacteria on the surface of the tissue rather than surviving within the wound. In depth analysis of OTU association with patient and clinical presentation characteristics is ongoing.

Canine raw meat diets and antimicrobial resistant *E. coli*: is there a link?

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Antimicrobial resistance is increasing amongst canine commensal and clinical bacteria. Risk factors for faecal carriage of AMR *Escherichia coli* have been reported, including the consumption of raw meat diets, however few studies have investigated dogs fed on such diets. This study aimed to determine the prevalence of AMR (resistance to at least one antimicrobial), multidrug resistant (MDR; resistance to three or more antimicrobial classes) and third generation cephalosporin resistant (3GCR) in canine faecal *E. coli*. The University of Liverpool Ethics Committee approved this study in March 2015. Faecal samples (n = 190) were obtained between May and July 2015 from dogs eating raw-meat (n = 114), or cooked meat (n = 76) diets. Selective and enrichment culture were used to detect bacteria and biochemical testing and PCR assays for the *uidA* gene were used to confirm identification. *E. coli* were tested for antimicrobial resistance by disc diffusion (CLSI 2013) to a range of antimicrobials (amoxicillin, amoxicillin-clavulanate, gentamicin, ciprofloxacin, chloramphenicol, tetracycline and trimethoprim sulfamethoxazole) and isolates from 3GCR impregnated agar plates (1 µg/ml ceftazidime and 1 µg/ml cefotaxime) were additionally tested for cefpodoxime resistance. AMR was significantly more likely to be detected in raw-fed compared to cooked-meat-fed dogs: 54% of dogs (95% CI: 45–64) compared to 17% (95% CI: 9–26) of dogs (P < 0.001). Furthermore MDR was also more likely in raw-fed 25% (95% CI: 17–32) compared to 4% (95% CI: 0–8) of cooked-meat-fed dogs (P < 0.001). 3GCR *E. coli* was detected in 31% (95% CI: 22–39) of dogs that were raw-fed and only 4% (95% CI: 0–8) of dogs that were cooked-meat-fed dogs (P < 0.001). Raw-fed dogs may be a source of antimicrobial resistant *E. coli* for the household representing both a potential public health and animal welfare issue. Preventative measures need to be implemented to prevent dissemination of such bacteria.

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Survival of extended-spectrum beta-lactamase producing *escherichia coli* in dog faeces

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Aims of study: The increasing prevalence of antimicrobial-resistance amongst bacteria poses significant problems for human and veterinary medicine. Production of extended-spectrum beta-lactamases (ESBLs) by bacteria such as *Escherichia coli* represents a particular concern; these enzymes confer resistance to a wide range of beta-lactam antimicrobials, including the critically important third-generation cephalosporins. Faeces from dogs with resistant bacteria may disseminate, transmit or propagate antimicrobial resistance. However, it is unknown how long such bacteria can survive in canine faeces and hence their risk in perpetuating antimicrobial resistance is uncertain. The aims of this study were to determine the survival numbers and times of ESBL-producing *E. coli* in dog faeces maintained at different temperatures and conditions, and to evaluate if survival of ESBL-producing *E. coli* differs from non-ESBL-producing, multidrug resistant (MDR) strains.

Methods: Freshly collected canine faecal samples were determined to be free of ESBL-producing and MDR *E. coli*. These were then separately inoculated with approximately 10⁶ CFU (colony forming units)/g of two previously obtained MDR and ESBL-producing *E. coli* strains retrieved from an archived culture collection. Samples were then stored in open plastic containers under three conditions: i) incubated at 23°C, ii) cold storage at 4°C and iii) outside under variable temperatures. Samples were removed at 0, 1, 3, 5, 7, 14 and 21 days for enumeration of bacterial numbers on eosin methylene blue agar (EMBA) containing relevant screening antimicrobials. Recovered isolates were confirmed as *E. coli* with the *E. coli* specific *uidA* PCR and tested for ESBL-production and multidrug resistance. Survival curves were plotted, with regression analysis performed on the linearized survival slopes and comparison of slopes by analysis of covariance (ANCOVA).

Results: All bacteria survived under all conditions for a minimum of 7 days, with both ESBL-producing and MDR *E. coli* surviving in high numbers (10⁵–10⁶ CFU/g) for at least 21 days under incubator and cold storage conditions. Bacteria were not detectable under outside conditions after 14 days for MDR *E. coli* and after 7 days for ESBL-producing *E. coli*. There was no significant difference in survival slopes between the two bacterial strains for each of the three conditions. However, there was a significant difference in the survival slopes for both ESBL-producing *E. coli* and MDR *E. coli* between the different conditions (P = 0.002 and P = 0.03 respectively).

Conclusions: These results demonstrate that survival of relatively high numbers of multidrug resistant and ESBL-producing *E. coli* is possible in canine faeces for moderately extended periods. There does not seem to be a significant difference in survival slopes between ESBL-producing and non-ESBL-producing multidrug resistant *E. coli* strains, but for both strains survival is decreased under outside conditions. Faeces of dogs carrying antimicrobial-resistant bacteria may have role to play in maintenance of antimicrobial resistance and appropriate handling of canine faeces is important to limit the dissemination of such bacteria.

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The influence of androgen receptor polymorphisms on the development of cruciate disease in Rottweilers

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Cruciate ligament disease (CLD) is commonly cited as one of the most common orthopaedic problems in dogs and the last few years has seen a shift in epidemiology of the disease from older mixed breeds dogs to young, large breed dogs such as the Rottweiler. Androgens are key influences on the rapid growth seen in large breed dogs and polymorphic regions in the canine androgen receptor have been linked with a number of diseases. This study investigated whether polymorphic regions in the canine androgen receptor influenced the development of CLD in 423 Rottweilers. The study also gathered epidemiological information about the 423 Rottweilers by questionnaire. There was no significant difference in length of androgen receptor and development of CLD in all dogs except female neutered dogs where there was a significant result ($P = 0.036$). Analysis of questionnaire data also found that neutering significantly increased the risk of dogs developing CLD ($P = 0.0002$). No significance was found between CLD and epidemiological factors such as weight, exercise and diet. This suggests that genetic factors could be of greater importance than epidemiological factors in developing CLD and warrants further investigation in Rottweilers and other large breed dogs, in particular neutered females.

Scratching that itch – elucidating the spinal cord injury which causes reflex “phantom” scratching in canine syringomyelia

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Aims of study: Syringomyelia (SM) is characterised by fluid filled cavities in the spinal cord. A classic sign of severe SM is a tendency to scratch towards one shoulder referred to as “phantom scratching”. Stimulation of neck skin induces a rhythmic scratching action of the ipsilateral limb. Although easy to describe, the mechanism behind this action is less easy to elucidate. A popular explanation is that the dogs experience allodynia (itch evoked by lightly touching the surrounding skin) or paraesthesia (a spontaneous or evoked sensation). However if affected dogs experience unusual sensations why do they make little or no skin contact? SM phantom scratching is similar to fictive scratch which develops a few months after transection of the caudal cervical spinal cord. In fictive scratch, stimulation of a skin receptive field results in a reflex scratching action due to hyperactivity of the scratching central pattern generator (CPG) i.e. neural circuits controlling a stereotyped sequence of muscle contractions. The similarity

to fictive scratch suggests commonality of neural pathways. In this project we investigate the neuroanatomical site that relates to phantom scratching. We first investigate the hypothesis that phenomenon of phantom scratching is associated with a large dorsolateral syrinx in the upper cervical spinal cord segments. We then looked for an association to damage in other areas of the cervical spinal cord and investigated the hypothesis that phantom scratching is not just associated with a dorsolateral syrinx but one that extends to the superficial dorsal horn (SDH).

Methods: Medical records from a two year period were searched for Cavalier King Charles Spaniels that had magnetic resonance imaging and diagnosis of clinical SM. The cohort was divided into SM with phantom scratching (19 dogs) and SM but no phantom scratching (18 dogs). The MRI studies were anonymised, randomised and viewed in EFILM™. For each transverse image the maximum perpendicular dimensions of the syrinx in each spinal cord quadrant was determined. Visual assessment was made as to whether the syrinx extended to the SDH.

Results: The study found that phantom scratching is associated with a large dorsolateral syrinx that extends to the SDH in the C3-C6 spinal segments (C2-C5 vertebrae). The study did not find an association to damage of other areas of cervical spinal cord.

Conclusion: Phantom scratching in the dog is associated with a large syrinx that extends to the SDH in the C3-C6 spinal segments. We propose that phantom scratching is due to damage to projection neurons in lamina I of the SDH with consequent reduced descending inhibition to the lumbosacral scratching CPG. Drugs affecting SDH targets may be useful for management of phantom scratching. If a dog has SM extending to the SDH then it is at risk for phantom scratching. If an itchy SM affected dog has no SDH involvement then alternative explanations for scratching (e.g. allergic skin disease) should be investigated.

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Forebrain conformation changes in Chiari-like malformation

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Aims of study: Chiari-like Malformation (CM) is a caudal fossa and craniocervical junction disorder resulting in cerebrospinal fluid pathway obstruction and variable syringomyelia (SM). Recent studies suggest that conformational changes are not confined to the hindbrain and that the entire skull base is foreshortened. Insufficient room for the forebrain may contribute to caudal displacement and overcrowding of the hindbrain. The olfactory bulbs (OB) are ventrally orientated in brachycephalic dogs; it has been suggested that this may be more extreme in CM. Recently genetic studies have

suggested a candidate gene *Sall-1* for canine CM. *Sall-1* deficiency in mice is associated with decreased OB size and defects in the human orthologue can be associated with skull abnormalities. We hypothesise Cavalier King Charles Spaniels (CKCS) with CM and SM have smaller and more ventrally orientated OB with rostral forebrain flattening. We compared OB size, angulation between the OB and the hard palate and two measurements that represent flattening of the rostral forebrain from 5 phenotypic groups.

Methods: Medical records from a 2 year period were searched for CKCS that had brain magnetic resonance imaging (MRI) and neurological assessment. The cohort was divided as follows: SM with phantom scratching (15 dogs); clinical SM (e.g. pain) but no phantom scratching (17 dogs); behavioural signs of pain with CM but no SM (25 dogs); CKCS with no SM and no behavioural signs of pain or scratching (13 dogs). In addition medical records were searched for dogs in the 5–15kg weight range with normal brain MRI (19 dogs including 5 brachycephalic). The MRI studies were anonymised, randomised and viewed in EFILM™. 5 measurements were taken from the T2W mid-sagittal brain MRI: the OB length and height (product represented OB size), angulation between the dorsal OB and the frontal lobe (bottom angle), angulation between the frontal and parietal lobes (top angle) and angulation between the OB and hard palate (OB angle).

Results: There was a trend for decreasing mean OB size with increasing CMSM phenotype severity (SM-scratchers < SM non-scratchers < CM-pain < CKCS-control < other breed control). However post hoc analysis, Bonferroni, indicated that only “other-breed-control” was significantly different from the CMSM groups but was not significantly different from “CKCS control”. ANOVA analysis for OB, bottom and top angle did not reveal a statistically significant difference between the groups however for OB angle there was an apparent separation between the control and CMSM groups suggesting a trend towards more ventrally orientated OB with increasing CMSM phenotype severity.

Conclusion: This study suggests that CM should be considered a more global brain and skull conformational disorder with features of extreme brachycephaly including smaller more ventrally orientated OB however further work is required and the measurement technique has been refined for future studies. We recommend that future studies into MRI conformation of CM and SM uses rigorous phenotyping based on clinical signs and age.

Funding: This project was funded by a 40th anniversary PetSavers Student Research Grant. The postgraduate research fees for SPK are funded by Cavalier Matters.

The impact of hypoxia on T regulatory cells in canine cancer and inflammation

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The presence of hypoxia and regulatory T cells (Tregs) in cancer has been associated with a poorer prognosis through treatment resistance and increased malignancy in some tumours. However, the precise relationship

between Tregs and hypoxia in canine tumours has still to be explored. The aim of this study was use immuno-histochemistry to detect expression of glucose transport-1 (GLUT1) and FoxP3 as respective markers for hypoxia and T regulatory cells, in benign and malignant tumours of different histotypes. Lymph node (LN) samples categorised as tumour-draining, metastatic or reactive due to inflammation were also examined. Both regulatory T cell and GLUT1 expression varied between tumour histotypes and LN types. There was an increased prevalence of FoxP3⁺ cells with increased GLUT1 labelling in all tumour and LN types, but metastatic LN results were confounded by strong GLUT1 labelling of some metastatic cells. This result suggests a possible link between hypoxia and Tregs in cancer and inflammation. Further research using additional markers for hypoxia and Tregs and a greater number of samples is warranted to explore potential novel therapeutic targets in the future.

Pilot study investigating the association between road traffic accidents and *Toxoplasma gondii* seroprevalence in cats

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Study Aim: It has been demonstrated that the parasite *Toxoplasma gondii* is able to alter the behaviour of rodents to show reduced cat aversion and therefore increase the likelihood of transmission from intermediate to definitive host. Similar behaviour changes have also been demonstrated within the human population, with changes in personality profiles and reaction times in infected individuals. Latent toxoplasmosis in the domestic cat is, however, generally considered to be asymptomatic, with clinical signs, including neurological, only rarely reported.

With the most frequent cause of domestic feline mortality in the UK being attributed to trauma (12.2%) and with 60% of those involving road traffic accidents, this study aimed to investigate a possible link between *T. gondii* infection in cats and an increased risk of involvement in traumatic accidents; specifically, road traffic accidents. The hypothesis being that cats that have been involved in a road traffic incident will be more likely to be seropositive for *T. gondii* antibodies, when compared to a control population of cats, which have not been associated with such trauma.

Methods: Residual blood samples were collected into plain brown serum gel tubes from two groups of felines: one group of which had been involved in a road accident (N=13) and the other being geographically matched controls (N=10). *Toxoplasma gondii* IgM and IgG antibody titres were detected using indirect immunofluorescence (IIFT). These groups were compared using the non-parametric Fisher's exact test. A *T. gondii* UK seroprevalence in domestic cats 'heat

map' was also generated from data (N=1344) obtained from UK commercial laboratories.

Results: There were no significant differences observed between these two groups in relation to seropositivity status ($P=1.0$). The prevalence 'heat map' indicated that seroprevalence may vary within the UK, confirming the need for geographically matched controls.

Conclusion: The study did not find a significant association between *T. gondii* infection and road accidents in cats. This study's major weakness to date was the low sample number, which made obtaining a significant, reliable answer to the stated hypothesis difficult. The study is ongoing and continues to collect samples in the hope that in the near future the study can be repeated with a larger cohort; therefore, this can be seen as an intermediate result.

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Urine as a potential source of biomarkers of canine spinal cord injury: challenges of biomarker discovery

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Urine is a source of potential biomarkers for various diseases but there is currently no consensus on how canine urine should be processed for proteomic analysis. This study investigated the effect of boric acid and centrifugation on urinary proteins. These steps are commonly recommended for urinary proteomic analysis, since boric acid inhibits bacterial growth in stored urine, while centrifugation helps to minimize cellular contamination of urine. The method employed to assay protein content is another consideration since agents present in urine may interfere with the reaction and generate erroneous values. This study therefore also compared the use of the bicinchoninic acid (BCA) assay and the Bradford assay to quantify urinary proteins. Random spot urine samples were collected from a control group (consisting of clinically healthy dogs and dogs with a variety of non-neurological conditions), and a group of dogs with spinal cord injury (SCI). On visual inspection, the intensities of protein bands visible on Coomassie blue-stained acrylamide gels were unaffected by centrifugation in the majority of samples analysed. However, samples positive for blood contamination (detected on dipstick) displayed a few unique bands and these bands were markedly reduced in intensity following centrifugation. Boric acid had no significant effect on the overall protein profile, however in 8 out of 24 samples, a protein band visible between 50–75 kDa decreased in intensity. The BCA and Bradford assays were used to calculate the volume required to give equal amounts of protein across all samples that were loaded onto the gel. Comparison of control and SCI samples showed that a number of SCI samples had a higher protein content compared to control samples on the gel based on the BCA assay values but displayed a comparable profile on the gel using the Bradford assay values. In conclusion, boric acid and centrifugation can

influence the level of specific proteins. Centrifugation is recommended for blood-contaminated samples in order to obtain an accurate assessment of the urinary proteome. The BCA and Bradford protein assays provide largely comparable results, however the BCA assay might be more susceptible to interfering substances in urine. Optimising the protocols for sample preparation and protein measurements are essential for validation of potential urinary biomarkers of disease.

Can alkaline phosphatase predict clinical control by trilostane of canine hyperadrenocorticism?

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Background: Alkaline phosphatase concentrations are increased in most cases of hyperadrenocorticism and remain increased despite trilostane treatment. Previously there have been no correlations found between alkaline phosphatase concentrations and post ACTH cortisol concentrations. Recent research however has shown that post ACTH cortisol concentrations are a poor measure, and pre-trilostane cortisol concentrations a better measure, of clinical control as assessed by an owner questionnaire.

Objective: To assess the correlation between serum alkaline phosphatase concentrations with pre-trilostane and the responses of owners to a validated questionnaire. Post trilostane cortisol concentrations (which are known to be poorly predictive of clinical control) were also compared as a negative control.

Methods: Two serum samples were obtained from each of 64 dogs being treated for hyperadrenocorticism using trilostane. The first serum sample was taken after admission (pre-trilostane). The trilostane dose was then administered, along with the dog's normal morning meal. A second serum sample was taken 3 hours later (post-trilostane). Cortisol concentration was measured concurrently on both serum samples using a competitive chemiluminescent enzyme immunoassay (Immulite®, Siemens). Alkaline phosphatase was measured in one of each group of samples using a validated in house analyser (Catalyst®; Idexx Laboratories). At the time of sampling each owner completed a previously validated questionnaire to assess the clinical control of the hyperadrenocorticism using nine questions. Each dog was then placed in a group according to its clinical control (excellent, moderate, poor). Dogs that were unwell for any reason were excluded from this study.

Results: The correlation coefficient between the ALP activity and the owner score ($r = -0.183$), pre trilostane cortisol ($r = 0$) and post-trilostane cortisol concentrations ($r = -0.04$) indicated that there was no linear relationship with any of these three measures of control of hyperadrenocorticism.

Conclusions: The very poor correlation between serum alkaline phosphatase concentration and both the pre-trilostane cortisol concentration and the owners scores exclude it as an accurate or reliable parameter to monitor the treatment of hyperadrenocorticism.